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Benzoxazin-4-ones as novel, easily accessible inhibitors for rhomboid proteases

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ABSTRACT

Rhomboid proteases form one of the most widespread intramembrane protease families. They have been implicated in variety of human diseases. The currently reported rhomboid inhibitors display some selectivity, but their construction involves multistep synthesis protocols. Here, we report benzoxazin-4-ones as novel inhibitors of rhomboid proteases with a covalent, but slow reversible inhibition mechanism. Benzoxazin-4-ones can be synthesized from anthranilic acid derivatives in a one-step synthesis, making them easily accessible. We demonstrate that an alkoxy substituent at the 2-position is crucial for potency and results in low micromolar inhibitors of rhomboid proteases. Hence, we expect that these compounds will allow rapid synthesis and optimization of inhibitors of rhomboids from different organisms.

Rhomboid proteases are amongst the most widespread intramembrane protease families, and have been found in virtually all sequenced organisms.[1,2] They utilize a serine-histidine dyad to cleave their substrates, which are also membrane proteins, in a transmembrane helix or a juxtamembrane region. Rhomboid proteases were originally discovered in *Drosophila melanogaster*.[3] In this organism, Rhomboid-1 is a vital player in the epidermal growth factor receptor pathway. Various biological roles in other organisms have been discovered since then, such as the mediation of quorum sensing by processing of a component of the twin arginine translocase in the bacterium *Providencia stuartii*[4] and ER associated degradation of membrane proteins.[5] More medically relevant roles of rhomboid proteases include cleavage of adhesins that help apicomplexan parasites invade host cells,[6,7] and processing of Pink-1, a protein involved in mitophagy and linked to Parkinson's disease.[8–10] Potent and selective inhibitors would be attractive research tools to examine these processes and assess the drugability of rhomboids.

After protocols had been established for the expression and purification of rhomboid proteases in detergent micelles,[11,12] several different types of inhibitors were discovered,[13,14] all of which contain an electrophile that reacts with the serine nucleophile in the active site. These inhibitors include 4-chloro-isocoumarins,[15,16] fluorophosphonates,[17,18] β-lactams,[19,20] β-lactones,[21] and peptidyl chloromethyl ketones,[22] aldehydes[23] and ketoamides (Fig. 1A).[24]

Whereas 3,4-dichloroisocoumarin (DCI) and the fluorophosphonate-based activity-based probe FP-Rh are pan-inhibitors of rhomboid proteases, β-lactones and other chloro-isocoumarins have shown some degree of selectivity.[25] During the course of this work, ketoamides were reported as a scaffold with selectivity against the *E. coli* protease GlpG over other, soluble serine proteases.[24]

Synthetically, most of these rhomboid inhibitors are not very easily accessible and require multistep organic synthesis. We therefore decided to seek for an inhibitor scaffold that would be easier to synthesize. Benzoxazin-4-ones (Fig. 1B) are heterocyclic, mechanism-based inhibitors of soluble serine proteases.[26] Attack on the carbonyl by the active site serine leads to acylation of the serine side chain, similar as with isocoumarins (Fig. 1C). Usually, the ester bond between protease and inhibitor hydrolyses over time, and the potency of benzoxazin-4-ones in part depends on the rate of deacylation (Fig. 1C).[26] Conveniently, benzoxazin-4-ones can be made in one synthetic step from substituted anthranilic acids and acid anhydrides, acid chlorides or chloroformates. The two fused aromatic ring structures in this class of inhibitors offer

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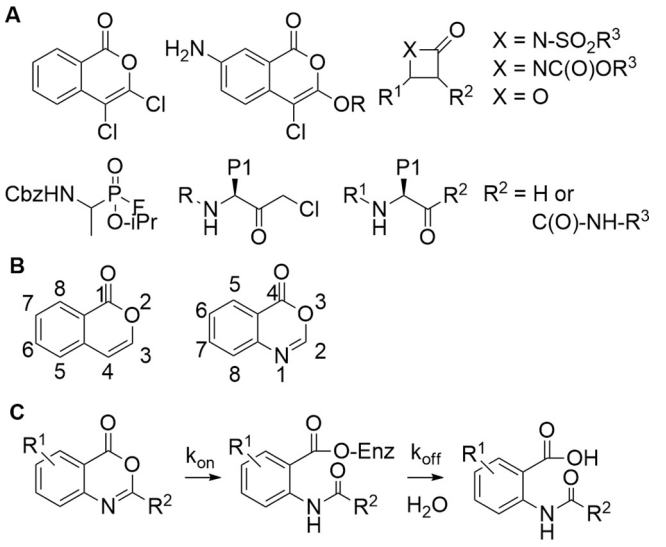
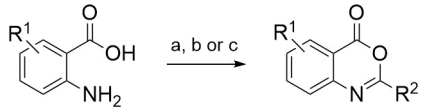


Fig. 1. (a) Overview of known rhomboid inhibitors. The general serine protease inhibitor 3,4-dichloroisocoumarin (top left), the 4-chloro-isocoumarin scaffold with a 7-amino group (top middle), β-lactams and β-lactones (top right), the fluorophosphonate CAPF (bottom left), peptide chloromethyl ketones (bottom middle) and peptide aldehydes and ketoamides (bottom right). (b) Comparison of the isocoumarin and benzoxazin-4-one scaffolds. (c) Mechanism of the inactivation of serine proteases by benzoxazinones and reactivation upon hydrolysis of the inhibitor acyl-enzyme complex.

the possibility of broad chemical variation and optimization for a particular target protease. Since the first report of benzoxazin-4-ones as serine protease inhibitors,[27] various publications have followed, for example on inhibition of human leukocyte elastase,[28,29] cathepsin G,[30] chymase,[31] and the complement system C1r serine protease.[32] We hypothesized that benzoxazinones may also inhibit rhomboid proteases, because of the structural analogy to 4-chloro-isocoumarins (Fig. 1B). From medicinal chemistry point-of-view, it is of interest to note that benzoxazinones with similar substitution pattern as 4-chloro-isocoumarins have a slightly lower ClogP (Supplemental Fig. 1). Rhomboid inhibitors of the 4-chloro-isocoumarin class carry small and large hydrophobic groups at the 3-position, which corresponds to the 2-position in the benzoxazin-4-one scaffold. We therefore selected a range of chloroformates and activated carboxylic acids to yield these type of substituents and increase the likelihood of obtaining potent rhomboid inhibitors.

We also chose a number of commercially available anthranilic acid derivatives to introduce variety at the aryl ring. For soluble serine proteases, the substituents at the aryl ring influence the rate of deacylation. It was reported that steric bulk at the 5-position slows down the deacylation step for human leukocyte elastase.[28] A 5-methyl group slows down this step for cathepsin G.[30] For HSV-1 protease, a 5-chloro substituent generally led to lower IC_{50} values.[33] We therefore chose the anthranilic acid precursors to include 5-chloro and 5-methyl groups, but we also selected a variety of other substituents to explore the influence on the potency against rhomboid proteases.



Scheme 1. Synthesis of benzoxazin-4-ones from anthranilic acids. *Reagents and conditions*: (a) Acetic anhydride, 130 °C, 86–87%. (b) Acid chloride, triethylamine, dichloromethane, 0 °C to room temperature, 13–98%. (c) Chloroformate, pyridine, room temperature, 6–57%.

Scheme 1 outlines the synthesis of the benzoxazinones we designed above. An overview of all synthesized compounds is given in Fig. 2. Compounds **1a**–**3a** were made from the corresponding anthranilic acid derivative and acetic anhydride under reflux conditions (Scheme 1). The other benzoxazinones were constructed by reacting the anthranilic acids with acid chlorides or chloroformates at 0 °C to room temperature, using triethylamine or pyridine as a base. All designed benzoxazinones were successfully synthesized, except for compound **1e**. The reason for this is unclear, as the close analogs **2e** and **3e** were both obtained in 86% yield.

Having the benzoxazinones at hand, we performed an inhibition assay against the *E. coli* rhomboid GlpG and the *B. subtilis* rhomboid YqgP by competitive activity-based protein profiling (ABPP). In competitive ABPP, protease samples are first treated with the compound to be tested and then with an activity-based probe (ABP) to label the remaining amount of active protease (Fig. 3A). Depending on the tag of the ABP, detection can take place in various manners. Here, we used FP-Rh, an ABP that reacts with the majority of all serine hydrolases[34] by means of its fluorophosphonate reactive group. It is also a suitable pan-rhomboid probe.[25] Hence, GlpG or YqgP were first incubated with the different benzoxazinone analogs for 30 min, and subsequently with FP-Rh for an additional 60 min, followed by SDS-PAGE. Whereas labeling of DMSO vehicle treated GlpG gave clear labeling by FP-Rh, the known isocoumarin inhibitor S006, which reacts with the active site serine and an additional histidine,[16] led to almost complete abrogation of the signal (Fig. 3B). To our delight, we found that several benzoxazinones also inhibited GlpG and YqgP (Fig. 3B and C).

For compounds that displayed lower than 5% residual activity in the initial screen, we determined the apparent IC_{50} value against the two rhomboids and two commercially available soluble serine proteases (Table 1). The potency against the two rhomboids is in the low micromolar to submicromolar range. Compound **2f**, the most potent benzoxazinone for GlpG, displays an apparent IC_{50} of 1.0 ± 0.64 μM. Benzoxazinone **5** turned out to be the most potent compound for YqgP with an apparent IC_{50} of 0.47 ± 0.16 μM. These potencies are comparable with those of the most potent 4-chloroisocoumarins[16] and β-lactams.[19,20]

From the series of compounds tested, we were able to derive a basic structure-activity relationship. The oxygen substituent on the 2-position seems to be crucial for the potency, as all compounds that showed low micromolar activity, carried an alkoxy substituent at the 2-position. Notably, compounds **2f** and **3f**, which both carry an OCH_2Ph substituent at the 2-position, display low micromolar activity, whereas the isosteric compounds **2h** and **3h**, which have a CH_2CH_2Ph group at this position, did not even make the cut-off in the general screen at 100 μM compound concentration (Fig. 3C), clearly indicating the requirement for the oxygen atom.

Interestingly, some benzoxazinones are able to discriminate between the different rhomboids. For example, **2f** inhibits GlpG with an IC_{50} value in the low micromolar range, while being only moderately active against YqgP, representing a selectivity of 15-fold. Compound **5** shows an apparent IC_{50} of 0.47 ± 0.16 μM for YqgP and approximately 60 μM for GlpG, corresponding to a selectivity of 130 fold. Since benzoxazinones have been reported as inhibitors of soluble serine proteases, we checked whether the rhomboid hits also inhibit two model serine proteases: bovine trypsin and chymotrypsin. We found that the benzoxazinones displayed higher potencies against these proteases than against rhomboids. Nevertheless, compound **3d** shows inhibition in the same order of magnitude for both rhomboids and soluble proteases. For compound **2d**, the potency for YqgP and trypsin is comparable. In an accompanying paper,[35] benzoxazinones with a 2-styryl substituent show some selectivity against rhomboids, but have lower potency than the here described compounds with a